ANNEX A

Netherlands

CHAPTER I

CONDITIONS FOR THE APPROVAL OF SEMEN COLLECTION CENTERS

Semen collection centers must:

(a) be placed under the permanent supervision of a center veterinarian;

(b) have at least

(i) animal housing including isolation facilities;

(ii) semen collection facilities including a separate room for the cleaning and disinfection or sterilization of equipment;

(iii) a semen processing room which need not necessarily be on the same site;

(iv) a semen storage room which need not necessarily be on the same site;

(c) be so constructed or isolated that contact with livestock outside is prevented;

(d) be so constructed that the animal housing and the semen collecting, processing and storage facilities can be readily cleaned and disinfected;

(e) have isolation accommodation which shall have no direct communication with the normal animal accommodation;

(f) be so designed that the animal accommodation is physically separated from the semen processing room and both are separated from the semen storage room.

CHAPTER II

CONDITIONS RELATING TO THE SUPERVISION OF SEMEN COLLECTION CENTERS

The collection centers must:

(a) be so supervised that they contain only animals of the species whose semen is to be collected. Other domestic animals which are strictly necessary for the normal operation of the collection center may nonetheless also be admitted, provided that they present no risk of infection to those species whose semen is to be collected and they fulfill the conditions laid down by the center veterinarian;

(b) be so supervised that a record is kept of all bovine animals at the center, giving details of the breed, date of birth and identification of each of the animals, and also a record of all checks for

diseases and all vaccinations carried out, giving also information from the disease/health file of each animal;

(c) be regularly inspected by an official veterinarian, at least twice a year, at which time standing checks on the conditions of approval and supervision shall be carried out;

(d) be so supervised that the entry of unauthorized persons is prevented. Furthermore, authorized visitors must be required to comply with the conditions laid down by the center veterinarian;

(e) employ technically competent staff suitably trained in disinfection procedures and hygiene techniques relevant to the control of the spread of disease;

(f) be so supervised that:

(i) only semen collected at an approved center is processed and stored in approved centers, without coming into contact with any other consignment of semen. However, semen not collected in an approved center may be processed in approved collection centers provided that:

- such semen is produced from bovine animals which fulfill the conditions laid down in Chapter I. 1(d) (i), (ii), (iii) and (v) of Annex B,

- processing is carried out with separate equipment or at a different time from semen intended for intra-Community trade, the equipment in the latter case being cleaned and sterilized after use,

- such semen may not be the subject of intra-Community trade and cannot at any time come into contact with or be stored with semen intended for intra-Community trade,

- such semen is identifiable by a marking different from that provided for in point (vii);

(ii) collection, processing and storage of semen takes place only on the premises set aside for the purpose and under conditions of the strictest hygiene;

(iii) all implements which come into contact with the semen or the donor animal during collection and processing are properly disinfected or sterilized prior to use;

(iv) products of animal origin used in the processing of semen - including additives or a diluent - are obtained from sources which present no animal health risk or are so treated prior to use that such risk is prevented;

(v) storage flasks and transport flasks are properly disinfected or sterilized before the commencement of each filling operation;

(vi) the cryogenic agent used has not been previously used for other products of animal origin;

(vii) each individual dose of semen is clearly marked in such a way that the date of collection of the semen and the breed and identification of the donor animal, as well as the name of the center, possibly in code, can be readily established; the characteristics and form of this marking will be established in accordance with the procedure laid down in Article 19.

ANNEX B

CHAPTER I

CONDITIONS APPLYING TO THE MOVEMENT OF ANIMALS INTO APPROVED SEMEN COLLECTION CENTERS

1. All bovine animals admitted to a semen collection center must:

(a) have been subjected to a period of isolation of at least 30 days in accommodation specifically approved for the purpose by the competent authority of the Member State, and where only other cloven-hoofed animals having at least the same health status are present;

(b) prior to their stay in the isolation accommodation described in (a) have belonged to herds:

(i) which are officially tuberculosis free;

(ii) which are officially brucellosis free or brucellosis free.

The animals may not previously have been kept in other herds of a lower health status;

(c) have come from a herd free of enzootic bovine leukosis or have been produced by a cow which has been subjected to a serological test for enzootic bovine leukosis with a negative result, not more than 30 days before the animal's admission to the center.

If this requirement cannot be fulfilled, the semen may not be the subject of trade until the donor has reached the age of two years and has been tested in accordance with Chapter II. 1 (iii) with a negative result;

(d) before the period of isolation specified in (a), and within the previous 30 days, have been subjected to the following tests with negative results:

(i) an intradermal tuberculin test carried out in accordance with the procedure laid down in Annex B to Directive 64/432/EEC;

(ii) a serum agglutination test carried out in accordance with the procedure laid down in Annex C to Directive 64/432/EEC and showing a brucella count lower than 30 IU of agglutination per milliliter and in the case of brucellosis free herds a complement fixation test showing a brucella count lower than 20 EEC units per milliliter (20 ICFT units);

(iii) a serological test for enzootic bovine leukosis carried out in accordance with the procedure laid down in Annex G to Directive 64/432/EEC;

(iv) a serum neutralization test or an Elisa test for infectious bovine rhinotracheitis/infectious pustular vulvo-vaginitis;

(v) a virus isolation test (fluorescent antibody test or immunoperoxidase test) for bovine viral diarrhoea. In the case of an animal less than six months of age the test must be deferred until that age is reached.

The competent authority may give authorization for the tests referred to in (d) to be carried out in the isolation accommodation, provided that the results are known before the commencement of the 30-day isolation period laid down in (e);

(e) during the period of isolation of at least 30 days specified in (a), have been subjected to the following tests with negative results:

(i) a serum agglutination test complying with the procedure laid down in Annex C to Directive 64/432/EEC and showing a brucella count lower than 30 IU of agglutination per milliliter and a complement fixation test showing a brucella count lower than 20 EEC units per milliliter (20 ICFT units) in the case of animals coming from brucellosis free herds;

(ii) either an immunofluorescent antibody test or a culture test for campylobacter foetus infection on a sample of preputial material or artificial vaginal washings; in the case of female animals a vaginal-mucus agglutination test shall be carried out;

(iii) a microscopic examination and culture test for trichomonas foetus on a sample of vaginal washings or preputial washings; in the case of female animals a vaginal mucus agglutination test shall be carried out;

(iv) a serum neutralization test or an Elisa test for infectious bovine rhinotracheitis/infectious pustular vulvo-vaginitis;

and have treatment against leptospirosis comprising two injections of streptomycin at an interval of 14 days (25 mg per kilogram of live body weight).

If any of the above tests should prove positive, the animal must be removed forthwith from the isolation accommodation. In the case of group isolation, the competent authority must take all necessary measures to re-establish the eligibility of the remaining animals for entry into the collection center in accordance with this Annex.

2. All tests must be carried out in a laboratory approved by the Member State.

3. Animals may only be admitted to the semen collection center with the express permission of the center veterinarian. All movements, both in and out, must be recorded.

4. No animal admitted to the semen collection center may show any clinical sign of disease on the day of admission. All animals must, without prejudice to paragraph 5, have come from isolation accommodation as referred to in paragraph 1 (a) which, on the day of consignment, officially fulfills the following conditions:

   (a) is situated in the center of an area of 10 kilometers radius in which there has been no case of foot-and-mouth disease for at least 30 days;

   (b) has for at least three months been free from foot-and-mouth disease and brucellosis;

   (c) has for at least 30 days been free from these bovine diseases which are compulsorily notifiable in accordance with Annex E to Directive 64/432/EEC.

5. Provided that the conditions laid down in paragraph 4 are satisfied and the routine tests referred to in Chapter II have been carried out during the previous 12 months, animals may be transferred from one approved semen collection center to another of equal health status without isolation or testing if transfer is direct. The animal in question must not come into direct or indirect contact with cloven-hoofed animals of a lower health status and the means of transport used must have been disinfected before use. If the movement from one semen collection center to another takes place between Member States it must take place in accordance with Directive 64/432/EEC.

CHAPTER II

ROUTINE TESTS AND TREATMENT WHICH MUST BE APPLIED TO ALL BOVINE ANIMALS IN AN APPROVED SEMEN COLLECTION CENTER

1. All bovine animals kept at an approved semen collection center must be subjected at least once a year to the following tests and treatment:

(i) an intradermal tuberculin test for tuberculosis, carried out in accordance with the procedure laid down in Annex B to Directive 64/432/EEC, with a negative result;

(ii) a serum agglutination test for brucellosis, carried out in accordance with the procedure laid down in Annex C to Directive 64/432/EEC, giving a count lower than 30 IU of agglutination per milliliter;

(iii) a serological test for enzootic bovine leukosis, carried out in accordance with the procedure laid down in Annex G to Directive 64/432/EEC, with negative result;

(iv) for infectious bovine rhinotracheitis/infection pustular vulvo-vaginitis, a serum neutralization test or an Elisa test with a negative result. However, until 31 December 1992, vaccination against these diseases may be practiced on sero-negative bulls, either with one dose of a temperature-sensitive live vaccine administered intranasally or two doses of an inactivated vaccine separated by an interval of not less than three weeks and not more than four weeks; the vaccination must be repeated subsequently at intervals of not more than six months;

(v) either an immunofluorescent antibody test or a culture test for campylobacter foetus infection on a sample of preputial material or artificial vaginal washings; in the case of female animals a vaginal mucus agglutination test must be carried out.

2. All tests must be carried out in a laboratory approved by the Member State.

3. If any of the above tests should prove positive, the animal must be isolated and the semen collected from it since the last negative test may not be the subject of intra-Community trade.

Semen collected from all other animals at the center since the date when the positive test was carried out shall be held in separate storage and may not be the subject of intra-Community trade until the health status of the center has been re-established.

ANNEX C

CONDITIONS WHICH SEMEN COLLECTED AT APPROVED CENTERS MUST SATISFY FOR THE PURPOSES OF INTRA-COMMUNITY TRADE

1. Semen must be obtained from animals which:

(a) show no clinical signs of disease on the day the semen is collected;

(b) (i) have not been vaccinated against foot-and-mouth disease; or

(ii) come from a center where all the animals have been fully protected against types A, O and C;

- and are thus either animals which, before entering the center, were not previously vaccinated against foot-and-mouth disease and must, therefore, have received two doses of inactivated virus vaccine approved and controlled by the competent authority of the exporting Member State at an interval of not less than six weeks and not more than eight months.

- or animals which, before entering the center, were vaccinated on at least three occasions at intervals of not more than one year.

When vaccination is practiced, all animals must receive repeat vaccinations at intervals of not more than 12 months;

(c) have not been vaccinated against foot-and-mouth disease within 30 days immediately prior to collection;

(d) have been kept at an approved semen collection center for a continuous period of at least 30 days immediately prior to collection of the semen;

(e) are not allowed to serve naturally;

(f) are kept in semen collection centers which have been free from foot-and-mouth disease for at least three months prior to collection of the semen and 30 days after collection, and are situated in the center of an area of 10 kilometers radius in which for at least 30 days there has been no case of foot-and-mouth disease;

(g) have been kept in semen collection centers which, during the period commencing 30 days prior to collection and ending 30 days after collection of the semen, have been free from those bovine diseases which are compulsorily notifiable in accordance with Annex E to Directive 64/432/EEC.

2. Antibiotics as listed below must be added to produce these concentrations in the final diluted semen:

not less than: 500 IU per ml streptomycin,
500 IU per ml penicillin,
150 ug per ml lincomycin,
300 ug per ml spectinomycin.

An alternative combination of antibiotics with an equivalent effect against campylobacters, leptospires and mycoplasmas may be used.

Immediately after their addition the diluted semen must be kept at a temperature of at least 5 C for a period of not less than 45 minutes.

3. Semen for intra-Community trade must:

(i) be stored in approved conditions for a minimum period of 30 days prior to dispatch;

(ii) be transported to the Member State of destination in flasks which have been cleaned and disinfected or sterilized before use and which have been sealed prior to dispatch from the approved storage facilities.

such semen may not be the subject of intra-Community trade and cannot at any time come into contact with or be stored with semen intended for intra-Community trade,

such semen is identifiable by a marking different from that provided for in point (vii);

(ii) collection, processing and storage of semen takes place only on the premises set aside for the purpose and under conditions of the strictest hygiene;

(iii) all implements which come into contact with the semen or the donor animal during collection and processing are properly disinfected or sterilized prior to use;

(iv) products of animal origin used in the processing of semen - including additives or a diluent - are obtained from sources which present no animal health risk or are so treated prior to use that such risk is prevented;

(v) storage flasks and transport flasks are properly disinfected or sterilized before the commencement of each filling operation;

(vi) the cryogenic agent used has not been previously used for other products of animal origin;

(vii) each individual dose of semen is clearly marked in such a way that the date of collection of the semen and the breed and identification of the donor animal, as well as the name of the center, possibly in code, can be readily established; the characteristics and form of this marking will be established in accordance with the procedure laid down in Article 19.

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November 1994

ANNEX B
PART 1

ANIMAL HEALTH CERTIFICATE
for importation of bovine semen from the United States of America

1. Consignor (name and full address)	Certificate number
	2. Third country of collection
3. Consignee (name and full address)	4. Competent authority
	5. Competent local authority
6. Place of loading	7. Name and address of semen collection center
8. Means of transport	10. Approval number of semen collection center
9. Place and Member State of destination	11. Seal number of semen containers
12. Identification of semen	fresh or frozen
	(* delete as appropriate)

(a) Number of doses	(b) Date(s) of collection	(c) Breed	(d) Identification of donor animal

13. I, the undersigned official veterinarian, have read and am familiar with Council Directive 88/407/EEC as amended and certify that:

(a)_____has been free from rinderpest and foot and mouth disease during the 12
(name of exporting country)

months immediately prior to collection of the semen for export and until its date of dispatch and no vaccination against these diseases has taken place during the same period;

(b) the semen collection center at which the semen to be exported was collected was:

(i) approved under the conditions laid down in Annex A, Chapter 1 of Council Directive 88/407/EEC;

(ii) operated and supervised under the conditions laid down in Annex A, Chapter II of Council Directive 88/407/EEC;

(iii) during the period commencing 30 days prior to the date of collection of the semen to be exported until 30 days after collection (in the case of fresh semen until the day of dispatch) have been free from rabies, tuberculosis, brucellosis, anthrax and contagious bovine pleuropneumonia;

(c) the bovine animals standing at the semen collection center:

(i) come from herds and/or were born to dams which satisfy the conditions of Annex B, Chapter I of Directive 88/407/EEC;

(ii) have, prior to entry into isolation, undergone the tests required by Annex B, Chapter I of Directive 88/407/EEC;

(iii) have satisfied the pre-entry isolation and testing requirements laid down in Annex B, Chapter I of Directive 88/407/EEC;

(iv) if resident for at least one year have undergone the routine tests according to Annex B, Chapter II of Directive 88/407/EEC;

(d) the semen to be exported:

(i) was obtained from donors which have been resident in

_____
(name of country)
for the period of six months immediately prior to collection of the semen for export and which satisfy the conditions laid down in Annex C of Directive 88/407/EEC;

(ii) was obtained from donors which are:

- standing in a semen collection center in which all the bovine animals have within the 12 months prior to collection of the semen for export been subjected with negative results to a serum neutralization test or an Elisa for infectious bovine rhinotracheitis/infectious pustular vulvo vaginitis (1),

or

- negative to a serum neutralization test or an Elisa for infectious bovine rhinotracheitis/ infectious pustular vulvovaginitis carried out within the 12 months prior to collection of semen for export (1),

or

- seropositive having been vaccinated in accordance with Annex B Chapter II of Council Directive 88/407/EEC and having given a negative result to a serum neutralization test or an Elisa carried out in the approved semen collection center prior to vaccination (1),

(1) Delete as appropriate.

or

- seropositive to a serum neutralization test or an Elisa for infectious bovine rhinotracheitis/infectious pustular vulvovaginitis, not vaccinated in accordance with Annex B Chapter II of Council Directive 88/407/EEC and at least 10% of each ejaculate of semen for export (with a minimum of five straws) has been subjected to an animal inoculation test or virus isolation test with negative results prior to export (1);

(iii) was processed, stored and transported under conditions which satisfy the terms of Directive 88/407/EEC.

(iv) was obtained from donor bulls which were subjected on two occasions not more than 12 months apart to the following precollection and post-collection tests with negative results in an approved laboratory (the post-collection test must be performed on a blood sample taken not less than 21 days following the collection of semen for export):

- a competitive Elisa for bluetongue in accordance with Annex B,

- an agar-gel immuno-diffusion test in accordance with Annex B and a virus neutralization test for all serotypes of epizootic haemorrhagic disease (EHD) known to exist in the exporting country.

Done at_____, on_____
(place) (date)

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Stamp Signature(2):_____
Name in block letters:_____
Official title:_____

(1) Delete as appropriate.
(2) The signature and the stamp must be in a color different to that of the printing.

ANNEX B

Protocol for a competitive Elisa (enzyme-linked immunosorbent assay), test using a group specific monoclonal antibody for the detection of bluetongue virus antibodies

THE BLUETONGUE COMPETITIVE ELISA USING MONOCLONAL ANTIBODY 3-17-A3

The test is capable of detecting antibodies to all known serotypes of bluetongue virus (BTV)

The principle of the test is the interruption of the reaction between BTV antigen and a group-specific monoclonal antibody (3-17-A3) by the addition of test serum dilutions. Antibodies to BTV present in the test serum block the reactivity of the monoclonal antibody (MAB) and result in a reduction in the expected color development on addition of enzyme substrate.

MATERIALS AND REAGENTS

1. Flat-bottomed microtitre plates.

2. Antigen: prepared as described below.

3. Blocking buffer: 5% (w/v) Marvel dried milk powder, 0.1 % (v/v) Tween-20 (supplied as polyoxyethylene sorbiton monolaurate syrup) in phosphate-buffered saline (PBS).

4. Monoclonal antibody: 3-17-A3 (supplied as hybridoma tissue-culture supernatant) stored at -20 degrees C or freeze-dried, diluted 1/50 with blocking buffer before use, directed against the group-specific polypeptide p7.

5. Conjugate: rabbit anti-mouse globulin (absorbed and eluted) conjugated to horseradish peroxidase and kept in the dark at 4 degrees C.

6. Substrate and chromogen: 0.2 gm of orthophenylene diamine (OPD) dissolved in a buffer consisting of 2.553 gm of citric acid and 4.574 gm of di-sodium hydrogenorthophosphate made up to 500 ml with distilled water, divided into 25 ml aliquots and kept in the dark at - 20 degrees C, with 12 ul/25 ml of hydrogen peroxide (30 % w/v) added immediately before use.

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HANDLE OPD WITH CARE - WHERE RUBBER GLOVES - SUSPECTED MUTAGEN.

7. 1 Molar sulphuric acid: 26.6 ml of acid added to 473.4 ml of distilled water.

REMEMBER - ALWAYS ADD ACID TO WATER, NEVER WATER TO ACID.

8. Orbital shaker.

9. Elisa plate reader (the test may be read visually).

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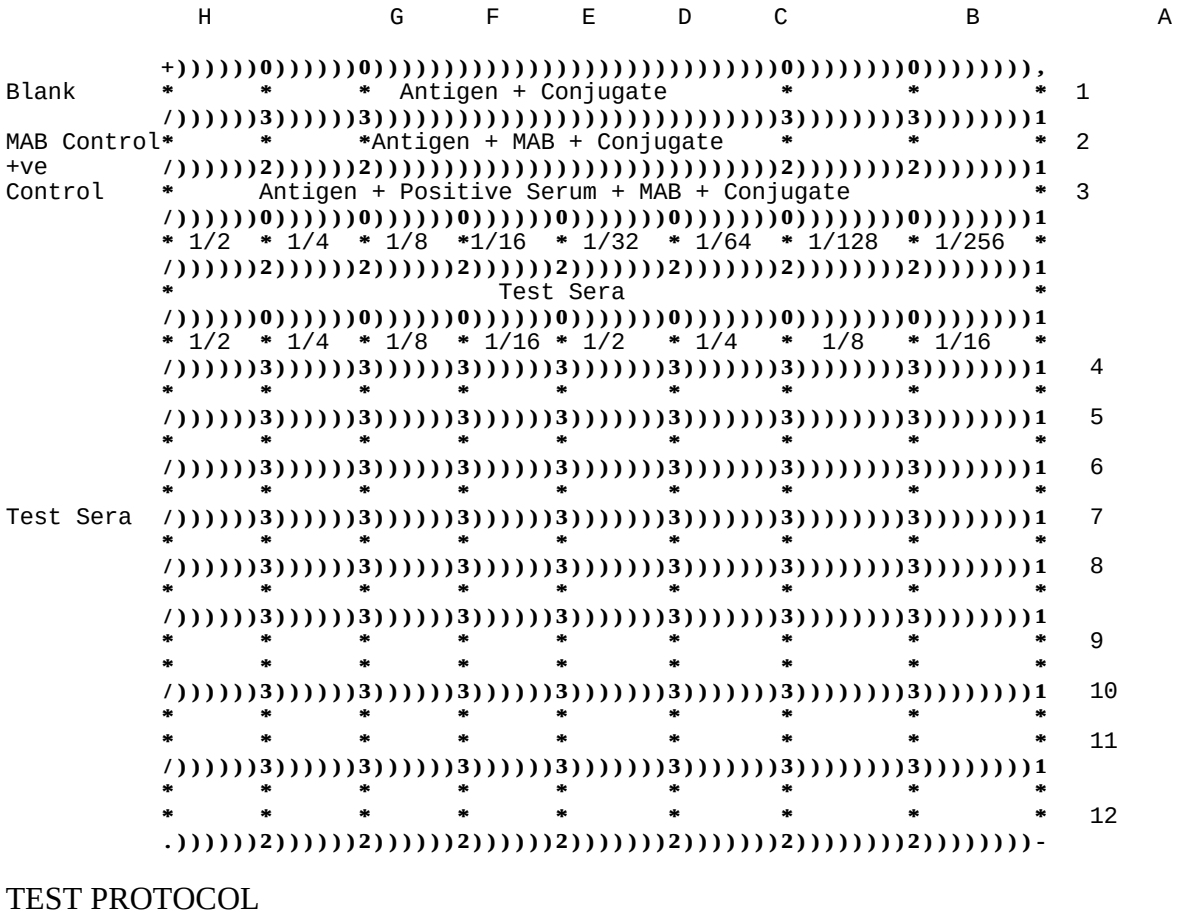
TEST FORMAT



TEST PROTOCOL

Blank control

Row 1 A - H is a blank control consisting of BTV antigen and conjugate. This may be used to blank the Elisa reader.

MAB control

Row 2 A - H is the monoclonal antibody control and consists of BTV antigen, monoclonal antibody and conjugate. This represents a negative control. The mean of the optical density readings from this control row represent the 0 % inhibition value.

Positive control

Row 3 A - H is the positive control. This consists of BTV antigen, BTV positive antiserum dilutions, MAB and conjugate. This is included as an indicator that the test is functioning properly and similar levels of inhibition should be obtained from test to test.

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Test sera

In the test format shown above, 18 sera can be tested over a dilution rate of 1/2, 1/4, 1/8 and 1/16. This will give some indication of the titre of antibody in the test sera. The dilution range could be extended further to obtain serum dilution end-point titres. Alternatively, for large-scale serological surveys, sera could be tested at a single dilution (1/4) or two dilutions (1/2 and 1/4) as a rapid screening test.

PROCEDURE

1. Dilute BTV antigen to pre-titrated concentration in PBS, sonicate briefly to dispense aggregated virus (if sonicator is not available, pipette vigorously) and add 50 ul to all wells of the Elisa plate. Tap sides of plate to disperse antigen.

2. Incubate at degrees 37 C for 60 minutes on an orbital shaker. Wash plates three times by flooding and emptying the wells with unsterile PBS and blot dry on absorbent paper.

3. Add 50 ul per well of blocking buffer. Add test sera and positive serum to the appropriate wells and dilute across the plate using a multichannel pipette. Do not add sera to the blank control, or the MAB control.

4. Immediately after the addition of the test sera, dilute MAB in blocking buffer (to pre-titrated dilution) and add 50 ul to all wells of the plate except for the blank control.

5. Incubate at 37 degrees C for 60 minutes on an orbital shaker. Wash three times with PBS and blot dry.

6. Dilute rabbit anti-mouse concentrate to 1/5000 in blocking buffer and add 50 ul to all wells of the plate.

7. Incubate at 37 degrees C for 60 minutes on an orbital shaker. Wash three times with PBS and blot dry.

8. Thaw the OPD and immediately before use add 12 ul of 30 % hydrogen peroxide to each 25 ml of OPD. Add 50 ul to all wells of the plate. Allow color to develop for approximately 10 minutes and stop the reaction with 1 M sulphuric acid (50 ul per well). Color should develop in the MAB control wells and in those wells containing sera with NO antibody to BTV.

9. Examine and record the plates either visually or using a spectrophotometric reader.

ANALYSIS OF RESULTS

Calculate the mean OD reading from the MAB controls. This represents the 0 % inhibition value. Optical density readings from the test sera are expressed as percentage inhibition values using the following formula:

$$\text{Percentage Inhibition Value} - 100 - \frac{\text{OD in the presence of test serum}}{\text{OD in the absence of test serum}} \times 100$$

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Inhibition values greater than 40 % at a serum dilution of 1/4 are considered positive. Visual reading is possible as 40 % inhibition is the lowest value easily discernible by eye.

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PREPARATION OF BTV ELISA ANTIGEN

1. Wash 10 roux of confluent BHK-21 cells three times with serum-free Eagle's medium and infect with bluetongue virus serotype 1 in serum-free Eagle's medium.

2. Incubate at 37 degrees C and examine daily for cytopathic effect (cpe).

3. When cpe is evident in 80 - 90% of the cell sheet of each roux, harvest the virus by shaking any still attached cells from the glass.

4. Centrifuge at 2,000 - 3,000 rpm to pellet the cells.

5. Discard the supernatant and resuspend the cells in approximately 30 ml of PHS containing 1 % 'Sarkosyl' and 2 ml phenolmethylsulphonyl fluoride (lysis buffer). This may cause the cells to form a gel and more lysis buffer may be added to reduce this effect.

NOTE: phenylmethylsulphonyl fluoride is harmful - handle with extreme caution.

6. Disrupt the cells for 60 seconds using an ultrasonic probe at an amplitude of 30 microns.

7. Centrifuge at 10,000 rpm for 10 minutes.

8. Store the supernatant at + 4 degrees C and resuspend the remaining cell pellet in 10 - 20 ml of lysis buffer.

9. Sonicate and clarify, storing the supernatant at each stage, at total of three times.

10. Pool the supernatant and centrifuge at 24,000 rpm for 120 minutes at + 4 degrees C over a 5 ml cushion of 40 % sucrose (w/v in PBS) using 30 ml Beckmann centrifuge tubes and an SW 28 rotor.

11. Discard the supernatant, drain the tubes thoroughly and resuspend the pellet in PBS by sonication. Store the antigen in aliquots at - 70 degrees C.

TITRATION OF BTV ELISA ANTIGEN

Bluetongue Elisa antigen is titrated by the indirect Elisa. Two-fold dilutions of antigen are titrated against a constant dilution (1/50) of monoclonal antibody 3-17-A3. The protocol is as follows:

PROCEDURE

1. Dilute BTV antigen in PBS across the microtitre plate in a two-fold dilution series (50 ul/well) using a multichannel pipette.

2. Incubate for 1 hour at 37 degrees C on an orbital shaker.

3. Wash plates three times with PBS.

4. Add 50 ul of monoclonal antibody 3-17-A3 (diluted 1/50) to each well of the microtitre plate.

5. Incubate for 1 hour at 37 degrees C on an orbital shaker.

6. Wash plates three times with PBS.

7. Add 50 ul of rabbit anti-mouse globulin conjugated to horseradish peroxidase, diluted to a pre-titrated optimal concentration, to each well of the microtitre plate.

8. Incubate for 1 hour at 37 degrees C on an orbital shaker.

9. Add substrate and chromogen as described previously. Stop the reaction after 10 minutes by the addition of 1 Molar sulphuric acid (50 ul/well).

In the competitive assay, the monoclonal antibody must be in excess; therefore, a dilution of antigen is chosen which fall on the titration curve (not on the plateau region) which gives approximately 0.8 OD after 10 minutes.

Protocol for an agar-gel immunodiffusion test for the detection of epizootic haemorrhagic disease antibodies

The agar-gel immo-diffusion test is carried out according to the following protocol:

MATERIALS AND REAGENTS

1. Antigen

Precipitating antigen is prepared in any cell culture system that supports the rapid multiplication of the appropriate serotype(s) of epizootic haemorrhagic disease virus. BKH or vero cells are recommended. Antigen is present in the supernatant fluid at the end of virus growth but requires 50 to 100 fold concentration to be effective. This may be achieved by any standard protein concentration procedure; virus in the antigen may be inactivated by the addition of 0.3 % (v/v) beta-propiolactone.

2. Known positive control serum

Using the international reference serum and antigen a national standard serum is produced, standardized for optimal proportion against the international reference serum, freeze-dried and used as the known control serum in each test.

3. Test serum

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PROCEDURE

1. 1% agarose prepared in borste or sodium barbitol buffer, pH 8.5 to 9.0 is poured into a petri dish to a minimum depth of 3.0 mm.

2. A test pattern of seven moisture-free wells, each 5.0 mm in diameter, is cut in the agar. The pattern consists of one center well and six wells arranged round it in a circle of radius 3mm.

3. The central well is filled with the standard antigen, Peripheral wells 2, 4 and 6 are filled with known positive serum; wells 1, 3 and 5 with test sera.

4. The system is incubated for up to 72 hours at room temperature in a closed humid chamber.

INTERPRETATION

A test serum is positive if it forms a specific precipitation line with the antigen and forms a complete line of identity with the control serum. A test serum is negative if it does not form a specific line with the antigen and it does not bend the line of the control serum. Petri dishes should be examined against a dark background and using indirect illumination.